

Immunostimulating activity of maysin isolated from corn silk in murine RAW 264.7 macrophages

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Corn silk (CS) has long been consumed as a traditional herb in Korea. Maysin is a major flavonoid of CS. The effects of maysin on macrophage activation were evaluated, using the murine macrophage RAW 264.7 cells. Maysin was isolated from CS by methanol extraction, and preparative C₁₈ reverse phase column chromatography. Maysin was nontoxic up to 100 µg/ml, and dose-dependently increased TNF-α secretion and iNOS production by 11.2- and 4.2-fold, respectively, compared to untreated control. The activation and subsequent nuclear translocation of NF-κB was substantially enhanced upon treatment with maysin (1-100 µg/ml). Maysin also stimulated the phosphorylation of Akt and MAPKs (ERK, JNK). These results indicated that maysin activates macrophages to secrete TNF-α and induce iNOS expression, via the activation of the Akt, NF-κB and MAPKs signaling pathways. These results suggest for the first time that maysin can be a new immunomodulator, enhancing the early innate immunity. [BMB Reports 2014; 47(7): 382-387]

INTRODUCTION

Corn silk (CS, *Zea mays* L.) has long been consumed in folk medicine to treat diverse symptoms, such as cystitis, edema, kidney stones, prostate disorders, urinary infections, and obesity (1-3). The predominant phenolic compounds in the methanol extract of CS have been identified as maysin, apimaysin (AP), 3'-methoxymaysin (ME), isoorientin, and luteolin derivatives (4, 5). Maysin (rhamnosyl-6-C-(4-ketofucoyl)-5,7,3',4' tetrahydroxyflavone), a flavone glycoside having a rhamnose,

is a major flavonoid in CS (Fig. 1A) (6). Although CS has long been consumed as a traditional medicine in Korea, the biological activities of maysin and other individual compounds in CS have not been well studied.

The stimulation of immune responses is regarded as one of the important strategies to enhance the body's defense systems in the elderly and cancer patients. As the front line of our immune response, macrophages play an important role in regulating innate and adaptive immune responses, by producing various types of cytokines and chemokines, and secreting cytotoxic and inflammatory molecules, such as NO and reactive oxygen species (ROS) (7, 8). The tumor necrosis factors-α (TNF-α), a pro-inflammatory cytokine, is a potent immunomodulator, derived from activated monocytes/macrophages and T lymphocytes (9, 10). Additionally, NO is also an important molecule in living organisms, as it is involved in the immune response against microorganisms (11). Recently, it was reported that generation of NO by iNOS is implicated in the regulation of apoptosis, and host defense against microorganisms and tumor cells (12). The development of effective immunomodulators that can activate macrophages is therefore crucial, in defending against microbial and environmental pathogens. Many studies have reported the immunostimulating activities of natural compounds or extracts, including flavonoids and polysaccharides (13-17).

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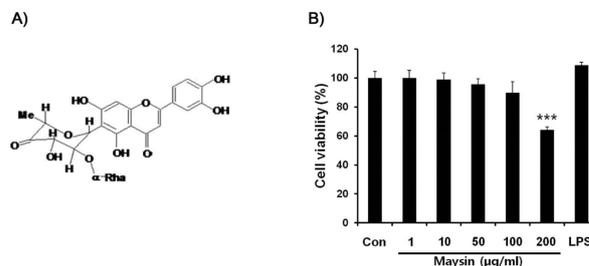


Fig. 1. Effect of maysin on the viability of RAW 264.7 cells. (A) Structure of maysin. (B) Cell viability was assessed by MTT assay, after treatment with maysin for 24 h. LPS (0.1 µg/ml) was used as a positive control. The data are expressed as the percent, normalized to the un-treated control (Con, 0 µg/ml). Data = mean ± SD, n = 3. ***P < 0.001, Student's *t*-test compared to the Con.

In this study, the immunomodulating activity of maysin, isolated from the CS of Kwangpyeongok, a Korean hybrid corn, was evaluated, using the murine macrophage RAW 264.7 cells. Herein we report that maysin activates murine RAW 264.7 macrophages to secrete TNF- α , and stimulate iNOS expression, by up-regulating the Akt, mitogen-activated protein kinases (MAPKs) and NF- κ B signaling pathways. This is the first study reporting on the immunomodulatory potential of corn silk maysin.

RESULTS

Cytotoxicity of maysin in RAW 264.7 cells

The effect of maysin on cell viability was determined by MTT assay. RAW 264.7 cells were incubated with maysin (1-200 μ g/ml), for 24 h. As shown in Fig. 1B, maysin did not affect the viability of RAW 264.7 cells, at concentrations up to 100 μ g/ml. However, a certain level of cytotoxicity of maysin was observed at higher concentrations; cell viability was affected by 35.8% (64.2% cell survival) at 200 μ g/ml. Thus, to avoid maysin-induced cytotoxicity, concentrations from 1 to 100 μ g/ml were selected for subsequent experiments.

Effect of maysin on TNF- α production and iNOS expression

The effect of maysin on TNF- α secretion was measured by ELISA. Maysin significantly induced TNF- α secretion, in a dose-dependent manner (Fig. 2A). Maysin (100 μ g/ml) was a weaker stimulator of TNF- α than LPS (0.1 μ g/ml), which was used as a positive control. However, whereas TNF- α production was not affected at concentrations below 1 μ g/ml, maysin significantly increased TNF- α levels at over 10 μ g/ml;

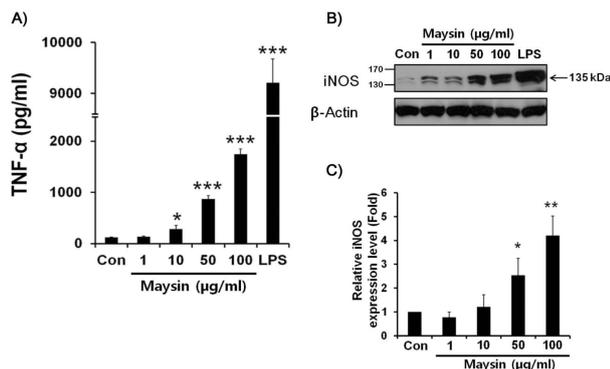


Fig. 2. Effects of maysin on the secretion of TNF- α and iNOS expression. (A) Cells were incubated with the indicated doses of maysin for 24 h. The levels of TNF- α in the culture supernatants were determined by ELISA. (B) Cells were collected after 24 h of treatment with maysin, and processed for Western blot analysis of iNOS. Actin was used as a loading control. (C) The data are expressed as the fold change, normalized to the Con. LPS (0.1 μ g/ml) was used as a positive control. Data = mean \pm SD, n = 3. *P < 0.05; **P < 0.01; ***P < 0.001. Student's *t*-test compared to the Con.

TNF- α production in the cells treated with 100 μ g/ml of maysin was approximately 14.74-fold higher, than un-treated control cells.

NO production via up-regulation of iNOS plays an essential role in the immune response (18). The cells were incubated with maysin (1-100 μ g/ml) for 24 h, and the expression levels of iNOS were determined by Western blot analysis. As shown in Fig. 2B and 2C, maysin significantly increased iNOS production in a dose-dependent manner. iNOS production was approximately 4.2-fold higher in maysin-treated cells (100 μ g/ml), compared to untreated cells.

Effects of maysin on the Akt/NF- κ B pathway

To examine the effects of maysin on the activation of the Akt (PI3K effector)/NF- κ B pathway in macrophages, the cells were treated with maysin (1-100 μ g/ml) for 24 h, and the levels of phosphorylated Akt and NF- κ B were determined by Western blot analysis. While the levels of total Akt (tAkt) were not affected, treating cells with maysin dose-dependently increased the levels of phosphorylated Akt, suggesting that maysin up-regulates the activation, but not expression, of Akt (Fig. 3A).

NF- κ B levels in the cytoplasmic and nuclear fractions of the cells treated with or without maysin were determined by Western blotting. While the expression levels of NF- κ B in the cytoplasmic fractions gradually decreased with increasing con-

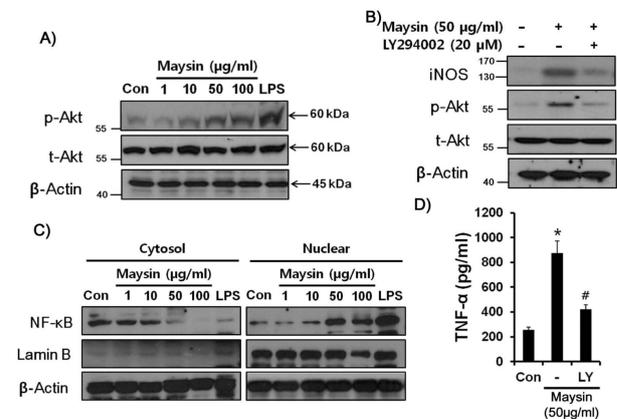


Fig. 3. Effects of maysin on Akt phosphorylation and NF- κ B activation in RAW 264.7 cells. (A) Whole cell lysates were collected, after treatment with increasing concentrations of maysin for 24 h. Total Akt (tAkt) was used as a loading control for the Western blot analysis of phosphorylated Akt (pAkt). (B) The levels of pAkt, tAkt, and iNOS were determined via Western blot analysis. (C) The activation of NF- κ B was assessed by Western blot analysis, using nuclear or cytosolic fractions. LaminB was used as a loading control for the nuclear fraction. LPS (0.1 μ g/ml) was used as a positive control. The cells were pre-treated with LY294002 (20 μ M) for 2 h, and then exposed to maysin (50 μ g/ml) for 24 h. (D) The levels of TNF- α in the culture supernatants were determined by ELISA. Data = mean \pm SD, n = 3. *P < 0.05, compared with the control; #P < 0.05, compared with the maysin-treated cells. Student's *t*-test compared to the control (Con).

centrations of maysin, the levels in the nuclear fractions increased in a dose-dependent manner, clearly indicating that maysin up-regulates the activation, and subsequent translocation of NF- κ B, from the cytosol to the nucleus (Fig. 3B). Additionally, pretreatment with specific PI3K inhibitor (LY294002) almost completely abolished the maysin-induced phosphorylation of Akt and expression of iNOS (Fig. 3C); and LY294002 also effectively decreased maysin-induced up-regulation of TNF- α secretion (Fig. 3D). Our data collectively demonstrated that maysin activates Akt/NF- κ B pathway, resulting in induction of iNOS expression and TNF- α secretion in RAW264.7 cells.

Effects of maysin on the activation of MAPK pathway

The MAPK signaling pathway, which includes extracellular activated signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), is known to participate in the immune response in macrophages (19). Therefore, the cells were treated with maysin (1-100 μ g/ml) for 24 h, and the levels of phosphorylated ERK1/2 and JNK were measured by Western blot analysis. Maysin induced the phosphorylation of both ERK1/2 and JNK, compared to the control. Whereas the total protein levels of ERK (t-ERK) and JNK (t-JNK) remained unchanged, the levels of phosphorylated ERK (p-ERK) in the cells treated with 50 and 100 μ g/ml of maysin gradually increased in a dose-dependent manner, compared to the un-treated control cells. Similarly,

phosphorylated JNK (p-JNK) levels in the cells treated with maysin were significantly higher, compared to the un-treated control group (Fig. 4A). When the phosphorylated proteins (p-ERK and p-JNK) were expressed as the ratios of the corresponding total proteins (t-ERK and t-JNK), 100 μ g/ml of maysin increased the phosphorylation of ERK1/2 and JNK by 1.4- and 3.3-fold, respectively, compared to the control (Fig. 4B, C). Furthermore, when the specific ERK (PD98059) and JNK (SP600125) inhibitors were pretreated, the maysin-induced phosphorylation of ERK and JNK and expression of iNOS were almost diminished (Fig. 4D and 4E), and these inhibitors also significantly blocked the maysin-induced up-regulation of TNF- α secretion (Fig. 4F). These results clearly suggested that ERK and JNK act at the upstream of iNOS expression and TNF- α secretion by maysin in RAW264.7 cells.

DISCUSSION

Although corn silk (CS) and its extracts have been consumed for a long time in folk medicine to treat diverse symptoms, and are currently marketed as a tea in Korea, reports on the biological and pharmacological activities of CS and its individual compounds are scarce. In this study, we attempted to evaluate the immunomodulatory activity of maysin, a major flavonoid in CS. The MTT assay showed that maysin does not stimulate the proliferation of murine macrophage cells; rather, it affects the cell viability, at over 200 μ g/ml. Maysin (up to 100 μ g/ml) dose-dependently activated the macrophages to secrete TNF- α and stimulate iNOS expression, which is responsible for the production of NO. Maysin also activated NF- κ B, and stimulated the phosphorylation of the upstream signaling components, Akt, ERK and JNK. Schepetkin et al. reported that a polysaccharide isolated from *Opuntia polyacantha* did not affect macrophage proliferation, but rather stimulated immunomodulatory activities, such as an increase in NO, TNF- α , IL-6 and NF- κ B levels (20).

Searching for new or more effective compounds that can potentiate immunological functions for immunopharmacological and oncotherapeutical purposes has become increasingly important. Activated macrophages produce various cytokines, chemokines, and cytotoxic and inflammatory molecules, such as NO and ROS (7, 8). TNF- α is a major pro-inflammatory cytokine produced in various immune cells, such as macrophages, monocytes, and T cells (9, 10), and the enhanced secretion of pro-inflammatory cytokines is a result of the immunostimulating effects of various compounds and extracts (8, 14). In our study, maysin was shown to stimulate TNF- α secretion in RAW264.7 cells. When macrophages are activated, the production of NO by iNOS is increased (18). The increase in NO production in immune cells by up-regulation of iNOS activity is an indication of immune activation (18). TNF- α and iNOS production is mediated by the PI3K/Akt signaling pathway, which is induced by natural compounds, such as polysaccharides (19). The Akt, which is activated by PI3K, is in-

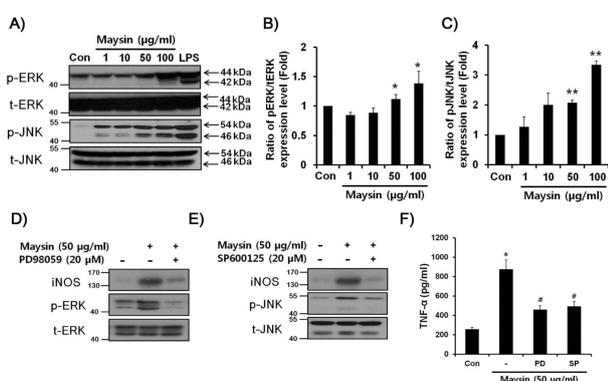


Fig. 4. Effects of maysin on the activation of ERK and JNK. (A) Cells were incubated with maysin, and the levels of phosphorylated ERK and JNK were then monitored by Western blot analysis. LPS (0.1 μ g/ml) was used as a positive control. The levels of p-ERK (B), and p-JNK (C), are expressed as the ratio of the phosphorylated sample to the corresponding total protein, according to densitometric analysis. Cells were pre-treated with PD98059 and SP600125 (20 μ M) for 2 h, and then exposed to maysin (50 μ g/ml) for 24 h. The level of phospho-activated forms of ERK and JNK, their total forms, and iNOS were determined via Western blot analysis (D and E), and the levels of TNF- α in the culture supernatants were determined by ELISA (F). Data = mean \pm SD, n = 3. *P < 0.05, **P < 0.01, compared with the control; #P < 0.05, compared with the maysin-treated cells. Student's *t*-test compared to the control (Con).

involved in modulating the activation of MAPKs, which in turn stimulates the production of various cytokines, including TNF- α and NO, in macrophages (19). Therefore, Akt has important functions in the immune system (21). NF- κ B is an important transcription factor in macrophage activation that regulates the transcription of many immunomodulatory mediators (15). NF- κ B is located in the cytosol, in quiescent cells. Upon activation, it is translocated into the nucleus, to initiate the transcription of target immune response genes, such as iNOS and TNF- α (19). Similarly, the results of our study also showed that maysin from CS enhanced Akt phosphorylation, and subsequently induced the translocation of NF- κ B into the nucleus, in RAW 264.7 cells (Fig. 3).

Many natural compounds have been reported to induce NF- κ B activation and the phosphorylation of MAPKs in macrophages, and the phosphorylation of MAPKs can activate the transcription of NF- κ B (15). Han et al. reported that flavonoids, kurarinone, and kuraridin affected iNOS and TNF- α production through MAPKs, including p38, ERK1/2 and JNK, and NF- κ B activation, in RAW264.7 macrophages (22). Therefore, our results strongly suggested that maysin from CS induces TNF- α and iNOS production, through the NF- κ B and Akt signaling pathways. MAPKs can be triggered by various extracellular stimuli, including LPS and other polysaccharides isolated from natural materials (23). It has been reported that MAPKs play important roles in the activation of NF- κ B, and subsequent pro-inflammatory cytokine expression (24). Similarly, our results demonstrated that corn silk maysin significantly increase the levels of phosphorylated ERK and JNK (Fig. 4). Taken collectively, the results of this study revealed that maysin has immunomodulatory effects, by increasing TNF- α secretion and iNOS production, via the activation of Akt, NF- κ B and MAPK (ERK and JNK) in RAW 264.7 macrophages, demonstrating for the first time that maysin from CS can be a new immunomodulator, for enhancing the early innate immunity.

In conclusion, this study showed that maysin, a major flavonoid isolated from the corn silk of Kwangpyeongok, a Korean hybrid corn, activates macrophages, and positively regulates the immune response. The underlying molecular mechanisms behind the maysin-induced activation of macrophages may involve the induction of TNF- α and iNOS, via up-regulation of the immune response-related Akt, NF- κ B and MAPKs (ERK and JNK) signaling pathways in macrophages. To the best of our knowledge, this is the first report on the immunomodulatory activity of corn silk maysin.

MATERIALS AND METHODS

Materials

RPML-1640 medium, fetal bovine serum (FBS), and antibiotics were purchased from GIBCO-BRL (Grand Island, NY, USA). The antibodies against phospho-Akt (phospho-Ser473, pAkt), total ERK (tERK), phospho-ERK (p-ERK), total tJNK, phos-

pho-JNK (p-JNK), NF- κ B p65, iNOS, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, and donkey anti-goat IgG-HRP, were obtained from Cell Signaling Technology (Danvers, MA, USA). The Lamin B and total Akt (tAkt) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The general signaling inhibitors, LY294002 (PI3K inhibitor), PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor), were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation and extraction of maysin

Kwangpyeongok, a Korean hybrid corn, was grown in a field at the National Institute of Crop Science, Suwon, Korea; and 3-5 day old silks were collected, with excising. The collected silks were immediately extracted with MeOH at 4°C for 1 week, and filtered through Whatman #4 paper. The filtrate was concentrated at 35-40°C, and the chlorophyll and lipids were removed by CH₂Cl₂ extraction. The final concentrate was subjected to preparative C₁₈ reverse phase column (25 mm × 54 cm) chromatography, under the pressure flow of 15 psi of nitrogen. After removing the sugars and water-soluble pigments, maysin was eluted from the column with 10%, 30%, and 50% MeOH, and evaporated to dryness. The crude maysin was dissolved in MeOH, and the silicic acid deposited mixture was subjected to silicic acid column (25 mm × 54 cm) chromatography, and was eluted with ethyl acetate. The ethyl acetate effluents were evaporated to dryness. Purified maysin was obtained, by performing C₁₈ reverse phase column (12.7 mm × 110 cm) chromatography. The endotoxin level in purified maysin was checked by chromogenic limulus amoebocyte lysate endotoxin assay kit (Genscript, Piscataway, NJ, USA), and resulted in the endotoxin content of less than 0.06 EU/ml (Endotoxin Unit/ml).

Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korea Cell Line Bank (Seoul, Korea), and grown in RPML-1640 medium (Gibco-Invitrogen, Grand Island, NY), supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin. Unless stated otherwise, the cells were incubated with varying concentrations (1 -200 µg/ml) of maysin, for 24 h. For the experiments with inhibitors, the cells were pretreated with the inhibitors (20 µM) for 2 h, and then incubated with 50 µg/ml maysin, for 24 h. Lipopolysaccharide (LPS) was used as a positive control, for activating the macrophages.

MTT cytotoxicity assay

The cytotoxicity of maysin was assessed, using the 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay. The cells (5 × 10⁴ cells/well), precultured in RPML-1640 media, were plated on 96-well microplates. Different concentrations (1-200 µg/ml) of maysin were prepared by serial dilutions. After 24 h of incubation, 20 µl of MTT (5 mg/ml) was added to each well, and the plates were

further incubated for 4 h. After the removal of the media, 200 μ l of DMSO was added into each well, to solubilize the formazan crystals. The absorbance was read at 570 nm, using a microplate reader (Molecular Devices Co., CA, USA).

Assay for TNF- α secretion

The concentration of TNF- α released from the cells treated with maysin was determined, using a Mouse TNF- α Enzyme-Linked Immunosorbent Assay (ELISA) kit (BD Biosciences, CA), according to the manufacturer's protocol. Briefly, the cells sub-cultured in RPMI-1640 media on 60 mm culture dishes were incubated for 24 h, with various concentrations of maysin. The culture supernatants were harvested, and TNF- α concentrations were determined, by measuring optical density.

Assays for NF- κ B activation

Nuclear extracts were obtained, according to a previous report, with minor modifications (25). Briefly, confluent cells in 10-cm-diameter dishes were resuspended in 200 μ l of buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT, 0.5 mM EDTA, 0.5 mM PMSF), and lysed with 12.5 μ l of 10% NP-40. The nuclear fractions were harvested by centrifugation at 14,000 rpm, at 4°C for 2 min. The supernatant was used as the source of the cytosolic proteins. The nuclear pellets were resuspended in 50 μ l of extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% NP-40), and then incubated on ice, for 10 min. The nuclear debris was removed by centrifugation at 14,000 rpm at 4°C for 10 min, and the supernatant was used as the source of the nuclear proteins. The activation and subsequent translocation of NF- κ B to the nucleus was determined by measuring NF- κ B protein levels in the nuclear and cytosolic extracts, by Western blot analysis.

Western blot analysis

Cell lysates were collected, by scraping the cells in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, pH to 7.8), containing protease and phosphatase inhibitor cocktails (Roche, Germany), and then centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were used for Western blot analysis. Protein concentration was measured, using the Bradford assay (26). The protein (30 μ g) was loaded onto 12% SDS-PAGE gels, and then transferred to nitrocellulose membranes. The membranes were incubated with the indicated primary antibodies, overnight at 4°C. The membranes were washed three times, incubated with horseradish peroxidase-conjugated antibodies for 1.5 h, and then visualized, using the enhanced chemiluminescence method (AbClon, Seoul, Korea). The relative levels of the proteins were quantified, using ImageJ software from the NIH (Bethesda, MA, USA).

Statistical analysis

The data from at least three independent experiments are ex-

pressed as the mean \pm S.D. Comparisons between two groups were performed using the unpaired Student's *t*-tests. A *P* value of less than 0.05 was considered statistically significant.

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